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FOREWORD

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INTRODUCTION

Paclitaxel (Taxol®) is an important agent in the treatment of breast cancer and other malignancies (1, 2). It is thought to stabilize microtubules resulting in mitotic block and cell death (3, 4). Its clinical utility is limited by the development of resistance. Initial studies identified the multidrug resistance (MDR) phenotype encoded by the *MDR1* gene with overexpression of the multidrug transporter P-glycoprotein (P-gp) providing a mechanism of resistance (5). Many chemotherapeutic agents, including paclitaxel are P-gp substrates, however, attempts to modulate *MDR1* have been minimally successful prompting the search for other mechanisms of resistance that may be clinically important.

Microtubules are complex, polymeric proteins composed of a backbone of tubulin heterodimers with α and β components and microtubule associated proteins (MAPs) (6-8). They are involved in numerous cellular processes (mitosis, cytoskeletal structure, axonal transport, motility).

Screening of DNA libraries has allowed identification of a number of β -tubulin isotypes in various species (6-10). Tubulin isotypes may be defined as tubulins which differ in their amino acid sequences prior to any post-translational modifications (phosphorylation and glutamylation). In humans, six isotypes, belonging to five classes β -tubulin have been described, grouped based on the carboxy-terminal amino acid composition (the most divergent portion between isotypes). While there is tissue specific isotype distribution, the data is mixed regarding the functional differences between the different isotypes. Certainly heterodimers incorporated into microtubules appear to interchangeably use the available β -tubulin isotype in a given cell.

There are more recent data suggesting phosphorylation differences, functional significance and possibly a role in drug resistance based on isotype composition. To date, alterations in *MDR1*, *p53*, *raf1 kinase*, *bcl-2*, tubulin isotype, and gene sequence have all been reported to alter sensitivity to tubulin poisons (11-18).

We have previously developed paclitaxel resistant uterine sarcoma cells by stepwise selection in paclitaxel (MES-SA-TP-30, MES-SA-T30). The variants selected paclitaxel alone (MES-SA-T30) show increased *MDR1* expression and the resistance is reversed by the addition of PSC-833. MES-SA-TP30 cells, selected with the P-gp competitive inhibitor PSC-833 (Valspodar) to suppress the emergence of *MDR1*, demonstrate stable paclitaxel resistance which is not affected by PSC-833. These resistant cells show elevated expression of the human β -9 tubulin isoform which has been previously associated with paclitaxel resistance. There are also alterations in the apoptotic proteins *BCL-2* and *BAX*. Given the species and tissue specificity of the different β -tubulin isotypes, we studied these proteins in human breast cancer cells.

With reference to the three tasks of this project, as noted in the prior report, we have completed task 1 (Development of resistance to taxol alone). This current report focuses on task 2 (Double selection of resistance to taxol and PSC 833) and task 3 (Investigation of taxol resistant mutants). We have developed cellular models of breast cancer cells resistant to both taxol and the combination of taxol with PSC 833, a potent inhibitor of *MDR1*. These cells demonstrate alterations in apoptosis regulating proteins as well as tubulin isotypes. The relationship of these changes to the resistance phenotype of these cells is currently being explored.

BODY

MATERIALS AND METHODS

Selection of paclitaxel resistant breast cancer cells

MDA-435 is a human breast carcinoma cell line obtained from ATCC. These cells were transfected with the murine ecotropic receptor by electroporation and selected in 1 mg/ml G418 in preparation for subsequent transfection and designated MDA-ECO. Cells selected with stepwise increasing concentrations of paclitaxel were serially passaged. Initially, cellular passage was complicated by the requirement of a minimal cell density for continued growth. Eventually cells were selected capable of surviving in 20nM paclitaxel (usual IC₅₀ about 1-2 nM) and were designated MDA-T20. A parallel selection was performed in the presence of 2 μ M PSC-833. The resulting cells are designated MDA-TP20. The cytotoxicity of various compounds incubated with and without modulators was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. IC₅₀ values (the drug concentration resulting in 50% inhibition of dye formation) were determined directly from semi-logarithmic dose-response curves.

Drugs

Paclitaxel and etoposide were obtained from Bristol-Myers Squibb Co. (Evansville, Ind.), vinblastine and vincristine from Eli Lilly and Co. (Indianapolis, IN) and doxorubicin from Adria Laboratories (Columbus, OH). All other cytotoxic agents were obtained from the National Cancer Institute (Bethesda, MD). [³H]-paclitaxel (19 Ci/mmol, 1 Ci/ml) was purchased from Moravek Biochemicals, Inc. (Brea, CA).

Amplimers used for reverse-transcriptase polymerase chain reaction (rt-PCR).

The oligonucleotides used as amplimers in this study were synthesized by Operon Technologies (Alameda, CA). Amplimers for *mdr1* were the following: 5' 3020-3037; 3' 3168-3187. We designed the following primers for analysis of the tubulin isotypes (Arabic numerals refer to the gene, Roman numerals refer to the tubulin protein isotype class):

- B α 1 forward primer: 5' (1003,1020) ATC AAG ACC AAG CGT ACC 3'
- B α 1 reverse primer: 5' (1363,1380) CAG CAC CTT TGT GAC GTT 3'
- K α 1 forward primer: 5' (1000, 1017) ACC ATC AAA ACC AAG CGC 3'
- K α 1 reverse primer: 5' (1363, 1380) TGC AGG GCC AAA AGG AAT 3'
- H α 44 forward primer: 5' (139, 158) CCT TCA CCA CCT TCT TCT GT 3'
- H α 44 reverse primer: 5' (230, 149) TCG GTA TGG GCC ATT TCG GA 3'
- M40 (class I) forward primer: 5' (-42,-22) CCA TAC ATA CCT TGA GGC GA 3'
- M40 reverse primer : 5' (226,246)GCC AAA AGG ACC TGA GCG AA 3'
- β 9 (class II) forward primer : 5' (1131,1150) CGC ATC TCC GAG CAG TTC AC 3'
- β 9 (class II) reverse primer : 5' (1301,1319) TCG CCC TCC TCC TCC TCG A 3'
- β 4 (class III) forward primer: 5' (1,15) ATG AGG GAA ATC GTG 3'
- β 4 reverse primer: 5' (223,243) AAA GGC CCC TGA GCG GAC ACT 3'
- 5 β (class IVa) forward primer: 5' (-85,-68) TCT CCG CCG CAT CTT CCA 3'
- 5 β reverse primer: 5' (167,186) TCT GGG GAC ATA ATT TCC TC 3'
- β 2 (class IVb) forward primer: 5' (-42,-22) GTC TAC TTC CTC CTC TTC CC 3'
- β 2 reverse primer: 5' (291,300) GTT GTT CCC AGC ACC ACT CT 3'
- γ forward primer: 5' (1055, 1072) AGT TGG CCA ACT TCA TCC 3'
- γ reverse primer: 5' (1349, 1367) TGC CCC AGG AGA TGT AGT 3'

The isotype classification used is the one described by Sullivan (6). These primers were designed using published sequence data for M40, 5 β and β 2 isotypes or, in the case of β 4 isotype a consensus forward primer and partial sequence information

generously provided by Kevin Sullivan (Scripps Research Institute, La Jolla, CA). Primers for M40, 5 β , β 2 and β 4 were designed to span introns. In the case of class II isotype, sequence was provided by screening expressed sequence tags from the EMBL GeneBank, using the peptide sequence previously reported by Cowan et al. (EST T03799) (9).

Reverse transcriptase polymerase chain reaction (rt-PCR)

The isolation of total RNA and rt-PCR were performed as previously described, with annealing at 55 °C and extension at 72 °C (19). Given the caveats of semi-quantitative PCR, we have tested each sample over a range of different number of PCR cycles and at different concentrations of cDNA. Ribosomal cDNA was used as an internal control for standardization and comparison of samples. The amplimers used for ribosomal RNA were the following: 5' 1501-1520); 3' 1846-1826. cDNAs were first adjusted in order to provide ribosomal PCR products which differed by less than 10%. PCR samples were analyzed by 8% polyacrylamide gel electrophoresis, stained with ethidium bromide, and analyzed by densitometric reading of bands on an Alpha Innotech IS-1000 image analyzer (San Leandro, CA).

RESULTS

Exposure of parental MDA-435-ECO cells to successively increasing concentration of paclitaxel resulted in variants demonstrating relative resistance (Table 1). These cells were maintained in the absence of paclitaxel and maintained their phenotype implying a genetic basis for their phenotype. The cytotoxicity to other chemotherapeutic agents demonstrated cross resistance to the vinca alkaloids in the MDA-T20 variant but not the MDA-TP20 variant. No cross resistance to either doxorubicin (an *MDR1* substrate) nor cisplatin (non-*MDR1* substrate) was observed.

Table 1. Cytotoxicity of cytotoxic agents in MDA variants measured by MTT assay in the absence of PSC-833. Expressed as IC₅₀. The paclitaxel data represents the results of 5 experiments over 4 months. Abbreviations are VCR-vincristine, VBL vinblastine.

<u>Cell Line</u>	<u>Paclitaxel</u>	<u>VCR</u>	<u>VBL</u>	<u>doxorubicin</u>	<u>Cisplatin</u>
MDA-ECO	2.3±0.6 nM	0.1 nM	0.45 nM	45 nM	12 nM
MDA-T20	19.4±2.7nM	0.95 nM	22 nM	40 nM	8 nM
MDA-TP20	14.2±3.2nM	0.085 nM	0.75 nM	30 nM	5.5 nM

Exclusion of P-glycoprotein mediated resistance

Since membrane transporters are a common mechanism of acquired resistance, we compared the parental and variant lines with regard to membrane transporters as a mechanism of the observed resistance. MTT cytotoxicity assays in the presence of the P-glycoprotein inhibitor PSC-833 showed no effect in the MDA-TP20 cells and slight sensitization in the MDA-T20 cells which was not surprising given the selection conditions. However, assessment of *MDR1* by rt-PCR showed lack of expression at the mRNA level in all three lines. Furthermore, intracellular accumulation assays using [3-H]-paclitaxel showed no difference between the three cell lines in the presence and absence of PSC-833 implying lack of a functional membrane transporter as a resistance mechanism in these cells.

Tubulins in Paclitaxel-Resistant Variants

We and others have demonstrated alterations in tubulins may confer paclitaxel resistance. Therefore we examined the parental and resistant lines for total tubulin content using pan-a and pan-b antibodies and saw no differences. Semiquantitative rt-PCR was performed on cellular preparations identically prepared from parental and variant cells in log growth phase. Primer pairs were as previously described for the following genes: b-tubulin isoforms (b1, b-2, M40, b-4, 5-b, b-9), *MDR1*, apoptotic proteins (*BCL-2*, *BAX*). Samples were analyzed by polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide and quantitated using an Alpha Innotech IS 1000 image analyzer. All reactions were normalized to ribosomal RNA. Using

previously defined isotype specific primers to assess β -tubulin isotype profiles in these variants we saw differences of uncertain significance (Table 2). Isotypes 5-b, β -4, and β -9 have been reported to be associated with paclitaxel resistance in certain model systems and there are slight alterations noted, but no changes more than 2-fold.

Table 2. Relative β -tubulin isotype expression in MDA variants as measured by rt-PCR using standard primers. (-) implies none detected within the limits of the assay.

Cell Line	M40	β -1	β -2	β -4	5- β	β -9	<i>MDR1</i>
MDA-ECO	1.47	-	2.22	1.75	1.68	2.30	neg
MDA-T20	1.34	-	2.88	2.34	2.25	2.8	neg
MDA-TP20	1.30	-	1.49	1.54	1.67	1.43	neg

Apoptotic Proteins in Paclitaxel Resistant Variants

There have been many recent reports of chemotherapy resistance secondary to failure of apoptosis. We therefore compared the cell lines with regard to *bcl-2* and *bax* by rt-PCR using standard primers and as shown in Table 3, there are increases in the anti-apoptotic *bcl-2* and decreases in the pro-apoptotic *bax* messages resulting in 6-7 fold

alterations in the *bcl-2* to *bax* ratio between the resistant selected variants and the parental control cells.

Table 3. Relative *bcl-2* and *bax* in MDA variants assayed by rt-PCR, normalized for ribo and relative to MDA-ECO.

Cell Line	<i>bcl-2</i>	<i>bax</i>	<i>bcl-2/bax</i> ratio
MDA-ECO	1.0	1.0	1.0
MDA-T20	3.67	0.49	7.5
MDA-TP20	2.75	0.4	6.9

DISCUSSION

We have developed stable cellular models of human breast cancer from the MDA-435 cell line that demonstrate non-MDR1 mechanisms of resistance to paclitaxel. The alterations in apoptotic proteins at the RNA level are of interest and we plan to exploit these models to better understand the steps leading to this phenotype (e.g. *raf1* kinase, *bcl-2* phosphorylation). Additionally, the cross resistance to vinca alkaloids (a class of drugs with activity in breast cancer) displayed in the MDA-T20 variant suggests alterations in tubulin properties (polymerization, binding sites, MAPs) that we plan to investigate further.

REFERENCES

1. Wall, M. E. and Wani, M. C. Camptothecin and taxol: discovery to clinic--thirteenth Bruce F. Cain Memorial Award Lecture, *Cancer Res.* 55: 753-60, 1995.
2. Gelmon, K. A. Biweekly paclitaxel (Taxol) and cisplatin in breast and ovarian cancer, *Semin Oncol.* 21: 24-8, 1994.
3. Caldas, C. and McGuire, W. P. Taxol in epithelial ovarian cancer, *Monogr Natl Cancer Inst* 155-9, 1993.
4. Parness, J. and Horwitz, S. B. Taxol binds to polymerized tubulin in vitro, *J Cell Biol.* 91: 479-87, 1981.
5. Horwitz, S. B., Cohen, D., Rao, S., Ringel, I., Shen, H. J., and Yang, C. P. Taxol: mechanisms of action and resistance, *Monogr Natl Cancer Inst* 55-61, 1993.
6. Sullivan, K. F. Structure and utilization of tubulin isotypes, *Annu Rev Cell Biol.* 4: 687-716, 1988.
7. Cleveland, D. W. and Sullivan, K. F. Molecular biology and genetics of tubulin, *Annu Rev Biochem.* 54: 331-65, 1985.
8. Luduena, R. F. Are tubulin isotypes functionally significant ?, *Mol Biol Cell.* 4: 445-57, 1993.
9. Cowan, N. J., Lewis, S. A., Sarkar, S., and Gu, W. Functional versatility of mammalian β -tubulin isotypes. *In*: R. Maccioni and J. Arechaga (eds.), *The Cytoskeleton in Cell Differentiation and Development*, pp. 157-66: ICSU Press, 1986.
10. Raff, E. C. The role of Multiple Tubulin Isoforms in Cellular Microtubule Function. *In*: J. S. Hyams and C. W. Lloyd (eds.), *Microtubules*, pp. 85-109. New York: Wiley-Liss, Inc., 1994.
11. Schibler, M. J. and Cabral, F. Taxol-dependent mutants of Chinese hamster ovary cells with alterations in α - and β -tubulin, *J Cell Biol.* 102: 1522-31, 1986.

12. Ohta, S., Nishio, K., Kubota, N., Ohmori, T., Funayama, Y., Ohira, T., Nakajima, H., Adachi, M., and Saijo, N. Characterization of a taxol-resistant human small-cell lung cancer cell line, *Japn J Cancer Res.* 85: 290-7, 1994.
13. Cabral, F. and Barlow, S. B. Mechanisms by which mammalian cells acquire resistance to drugs that affect microtubule assembly, *Faseb J.* 3: 1593-9, 1989.
14. Cabral, F., Sobel, M. E., and Gottesman, M. M. CHO mutants resistant to colchicine, colcemid or griseofulvin have an altered β -tubulin, *Cell.* 20: 29-36, 1980.
15. Haber, M., Burkhart, C. A., Regl, D., Madafiglio, J., Norris, M. D., and Horwitz, S. B. Taxol resistance in J774.2 cells is associated with altered expression of specific β -tubulin isotypes, *Proc Am Assoc Cancer Res.* 36: 318, 1995.
16. Minotti, A. M., Barlow, S. B., and Cabral, F. Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin, *J Biol Chem.* 266: 3987-94, 1991.
17. Boggs, B. A., Gonzalez-Garay, M. L., O'Brien, W. F., Barlow, S. B., and Cabral, F. Mechanisms of resistance to drugs that target microtubules, *Cell Pharmacol.* 1 (*Suppl 1*): S95-S101, 1993.
18. Cabral, F. and Barlow, S. B. Resistance to antimitotic agents as genetic probes of microtubule structure and function, *Pharmacol Ther.* 52: 159-71, 1991.
19. Jaffrezou, J. P., Chen, G., Duran, G. E., Kuhl, J. S., and Sikic, B. I. Mutation rates and mechanisms of resistance to etoposide determined from fluctuation analysis, *J Natl Cancer Inst.* 86: 1152-8, 1994.